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Release of Adenosine Triphosphate by Adenosine Diphosphate in Whole Blood and in Erythrocyte Suspensions

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In whole blood samples from thrombocytopenic patients, large amounts of ATP were released by ADP, exceeding the level obtained with samples from normal persons by far. Because we suspected that the high potential of ATP in erythrocytes would be the main source for this phenomenon, the release of ATP by ADP was measured in whole blood samples from normal, thrombocytopenic, and leukocytopenic persons and in suspensions of washed erythrocytes. The release was recorded by a Whole Blood Lumi-Aggregometer type 500 VS (Chrono-Log Corporation, Havertown, PA) using the luciferin-luciferase system. Not only in samples from thrombocytopenic persons but also with normal platelet count, increasing amounts of ATP were released with increasing ADP concentrations, finally exceeding the ATP releasable from thrombocytes by thrombin. The amounts of ADP required to match the ATP release of thrombin were closely correlated with the platelet counts in the samples. With lower platelet counts, the release mechanism from erythrocytes could be stimulated more easily by low concentrations of ADP. The binding of ADP to platelets occurred with ostensibly higher affinity. The phenomenon of overshooting ATP release was also observed in samples from extremely leukocytopenic patients. A very large release of ATP was also achieved in suspensions of washed erythrocytes. In this way our hypothesis of ATP release from erythrocytes by ADP was confirmed again. The mechanism of the release from erythrocytes remains unclear. We speculate that its purpose is to regulate extracellular nucleotides in the circulating blood. Am. J. Hematol. 56:259–265, 1997.

Key words: adenine nucleotides; erythrocyte; purinergic receptors; luciferin

INTRODUCTION

Adenosine diphosphate (ADP) induces shape change, aggregation, and exposure of fibrinogen binding sites on platelets. These activation processes may greatly contribute to hemostasis and thrombosis under physiological and pathological conditions. Adenine nucleotides originate in the extracellular space of the blood from different sources [1]. Erythrocytes are a very important source for ADP and adenosine triphosphate (ATP) [2]. ADP and ATP are also released by skeletal and heart muscle, the nervous system, and the blood vessel wall. One of the most important sources are the platelets themselves. The extracellular nucleotides are destroyed by enzymes in the plasma or by ectoenzymes located on the surface of blood cells [3,4] or endothelial cells [5–7]. The clearance process is essentially performed by erythrocytes, but only few reports exist about ADP converting enzymes on the surface of erythrocytes [1,8–11]. One of these is adenylate kinase, a 22.4-kDA soluble protein [8,10,12]. It is not firmly bound to the erythrocyte membrane but catalyzes the simultaneous transformation of ADP to ATP and adenosine monophosphate (AMP). In addition, there is an ecto-ADPase in association with the cell surface [11]. The main product from its activity is AMP. The ecto-ATPase is located in close proximity to the cell surface. Extracellular ATP can be converted by these ectonucleotidases in a rapid manner to AMP without accumulation

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of ADP. In plasma, ATP can be converted to AMP directly by a pyrophosphohydrolase [1].

ATP, AMP, and adenosine are well-known inhibitors of the ADP-induced stimulation of platelets [13]. ATP acts as a competitive inhibitor of ADP on the receptors, but also as a direct inhibitor of adenylate cyclase via the receptors. Furthermore, ATP influences platelet activation, stimulating the release of nitric oxide and prostacyclin from endothelial cells [14]. In this way adenine nucleotides may well control thrombosis and hemostasis. These pathogenetic aspects have been greatly neglected until now.

In our studies on the release reaction in whole blood induced by ADP, a strange phenomenon was observed that sheds new light on and poses new questions about these complicated relations. In whole blood samples from thrombocytopenic patients, large amounts of ATP were released by ADP, greatly exceeding the levels to be expected from platelets alone [15,16]. Starting from this observation, the aim of the study was to test the release of ATP from red cells, the results of which are reported here.

MATERIALS AND METHODS

Citrated whole blood was collected from 38 persons with normal platelet counts and from 20 thrombocytopenic patients (thrombocyte counts < 150,000/μl) by venipuncture in 3.8% trisodium citrate (1 part citrate plus 9 parts blood). Five of the thrombocytopenic patients were extremely leukocytopenic (leukocyte counts < 1,000/μl). The samples were stored for at least 30 min at room temperature. During this time, erythrocyte and platelet counts were performed on electronic counters. To obtain platelet-poor suspensions of erythrocytes in four samples from healthy persons, a special centrifugation procedure was performed. Platelet-rich plasma was separated by light centrifugation. Thereafter, the erythrocyte column was resuspended in a washing solution consisting of 3.8% trisodium citrate solution and isotonic saline in a ratio of 1:9. The suspension was centrifuged again and the supernatant removed. The washing process was repeated three times. After that the preparation was resuspended by the same solution to the original erythrocyte concentration. In every case the platelet count was decreased to values below 10,000/μl, but the leukocyte content cannot be diminished by this simple procedure. In two additional samples, low temperature preserved erythrocytes were tested. They had been prepared for transfusion and contained neither platelets nor leukocytes. Sometimes the free ATP content of the erythrocyte suspensions was higher than the free ATP content in whole blood specimens. The initial deflection for free ATP was subtracted from the peak height of the following release curve in every case.

The release of ATP was measured with a Whole Blood Lumi-Aggregometer type 500 VS (Chrono-Log Corporation, Havertown, PA) using the luciferin-luciferase system. The luminescence evoked by the luciferin oxidation was recorded photometrically after amplification by a photomultiplier. The sensitivity for ATP in whole blood samples was in the nmol range. We used the luciferin-luciferase reagent produced by the Chrono-Log Corporation (Chrono-Lume reagent) dissolved according to the producer's direction. As inducer of the release reaction, ADP at final concentrations of 5.5 to 60 μM from the same manufacturer (Chrono-Par reagent) was applied. It was dissolved according to the manufacturer's instruction in isotonic saline in such a manner that the addition of 1-μl solution to the sample yielded a final concentration of 1 μM. Chrono-Lume ATP (Chrono-Log Corporation) served as the standard in all experiments. It was dissolved so that 5 μl of the solution contained 2 nmol ATP.

The test procedure was as follows: 450 μl whole blood or red cell suspension was mixed with 450 μl isotonic saline in plastic cuvettes and prewarmed in the apparatus for at least 20 min. Thereafter, 100 μl of the luciferin-luciferase reagent was added. Shortly before this, stirring was started by a stir bar with a velocity of 1,000 rpm. The system was closed now for a short time to register the first deflection caused by the free ATP in the probe. After 2 min the system was opened, ADP was added, and the chamber closed again. The curves were registered at a speed of 1 cm/min. They were evaluated by measuring the height of their peak. In every case the release reaction by thrombin (Chrono-Log Corporation) was recorded for comparison. Maximal ATP release was achieved with final thrombin concentrations between 0.3 to 0.5 U/ml.

The following additional investigations were performed. The test procedure described above was carried out in erythrocyte suspensions without any inducing agent as well as with arachidonic acid (1.25 and 1.75 mM final concentration) and collagen (3 μg/ml) in samples of 3 individuals. ATP and ADP were tested in saline by the luciferin-luciferase system. For measurements in the aqueous medium, the ATP standard was diluted to 0.02 nmol in 5 μl. The original ADP solution was diluted 1:10 for this purpose. Further handling was the same as with blood specimens. Hemoglobin was detected in the supernatants of 2 whole blood specimens after incubation with different amounts of ADP (final concentrations 0–75 μM) at room temperature without stirring and in two other blood samples treated by the regular test procedure in the aggregometer with preincubation at 37°C, stirring, addition of the luciferin-luciferase reagent and the ADP solution (final concentration 0–60 μM). All specimens were centrifuged after a reaction time of 5 or 10 min with a velocity of 4,000 rpm for 30 min. The measurement was performed by aid of an automatic analyzer for he-

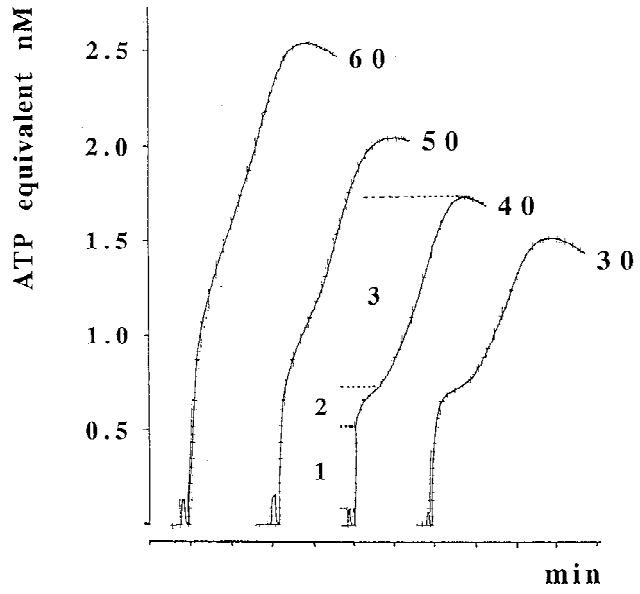

Fig. 1. ATP release by increasing concentrations of ADP (30–60 μM). The release curves obtained with whole blood samples were triphasic (1–3) and increased with the dose of the inducer.

moglobin (Diamat System Bio-Rad Laboratories, Munich, Germany). The lower sensitivity limit was below 10 nM and in a second set of experiments below 100 nM. This was tested by a gradually diluted hemoglobin solution. Lactate dehydrogenase was tested in supernatants of 13 samples of whole blood or erythrocyte suspensions after incubation with ADP in the same manner with and without regular stirring in the apparatus using the kit LDH opt (Boehringer Mannheim GmbH, Mannheim, Germany).

The correlation coefficients according to BRAVAIS and the coefficients for regression were calculated [17].

RESULTS

In whole blood samples obtained from healthy persons ADP induced triphasic release curves (Fig. 1). The phases were measured separately and named P1, P2, and P3. P1 designates a very steep ascent of the curve and is visible in all curves. The first deflection before addition of the inducer was subtracted in every case. It is caused by the free ATP amounts and was higher in suspensions of washed erythrocytes, especially in the preparations for the clinical use. They must be taken as the sequelae of cells damage by the handling of these samples. P2 was visible only with low inducer concentrations and disappeared with its increase. P1 was closely correlated with the sum of P2 and P3 ($r = 0.8866$; $n = 85$, $P < 0.001$).

As will be shown in Figure 4, the release curves increased steadily. With suspensions of washed red cells release reactions could also be obtained (Fig. 2). However, the curves exhibited another shape, two- or monophasic only. They were initiated by very low ADP concentrations. Initially we found that the high deflections declined in the course of the investigation before the addition of the inducer. ADP was added always after constant intervals. The ADP effect was independent of the baseline level. A marked release of ATP was observed from low temperature preserved and repeatedly washed erythrocytes by ADP as well (Fig. 3). Among other inducers, the most extensive release reaction from platelets in normal blood was achieved by thrombin. But with higher ADP concentrations this standard was considerably exceeded. Abundant amounts of ATP were released in the mixture under these conditions. The process seemed to be very large in our sensitive system.

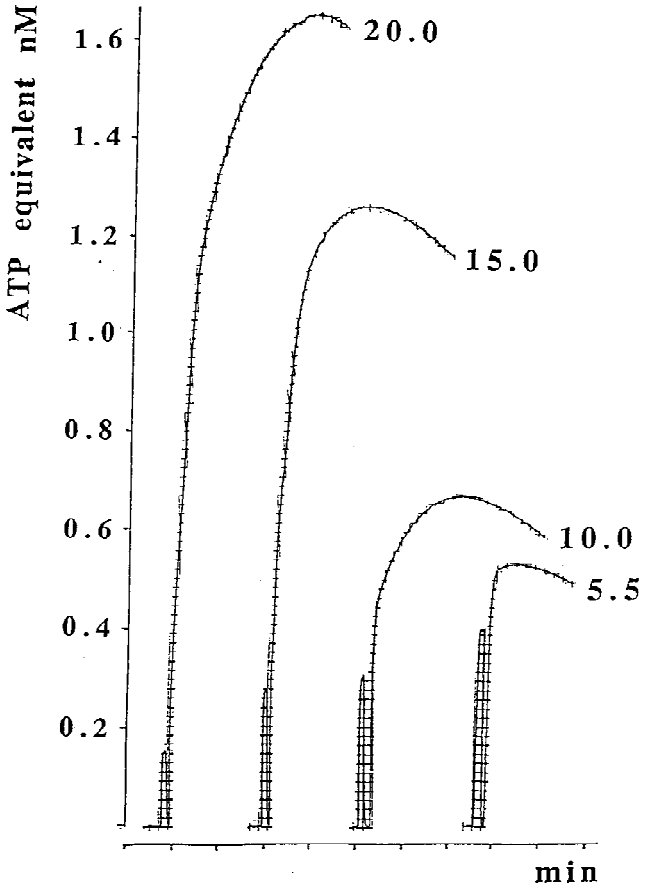

Fig. 2. ATP release in suspensions of washed red cells by ADP (5.5–20 μM, final concentrations). The release increased with higher inducer concentrations.

In the following experiments, the maximal release of ATP from thrombocytes by thrombin was ascertained in samples from persons with different platelet counts. The maximal ATP release by thrombin correlated very closely with the platelet count (Table I). Thereafter, the ATP release was recorded with different ADP concentrations (Fig. 4). The intention was to find the ADP concentration, which provoked the same release as an opti-

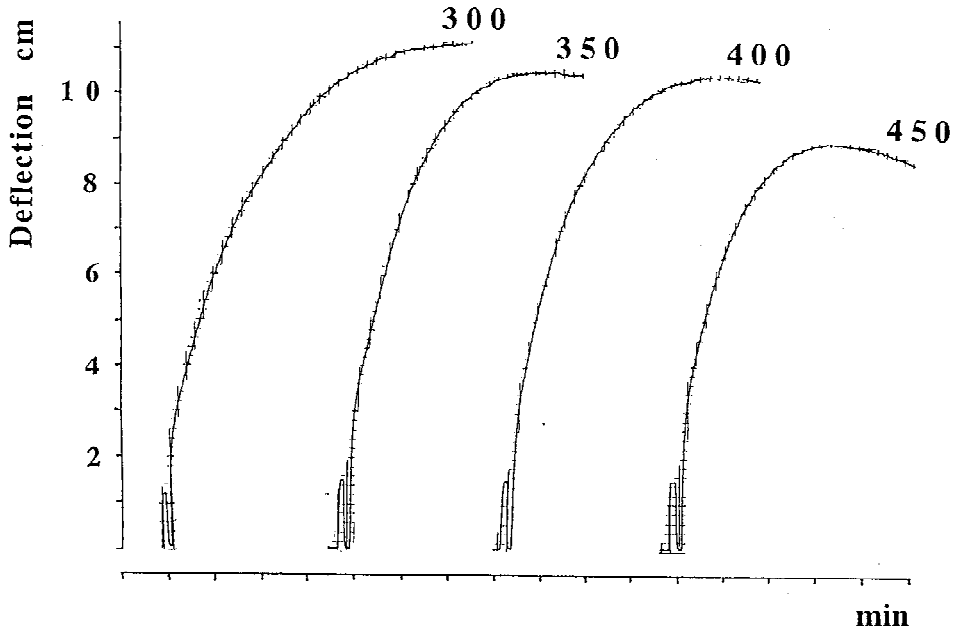

Fig. 3. Release of ATP from low temperature preserved erythrocytes by ADP. The numbers designate the volume of the red cell concentrate (erythrocyte count 6.28 × 10^{12}/l) in µl within the cuvettes. The peaks increased with decreasing red cell concentrations. The final concentration of ADP in all samples was 30 µM.

TABLE I. Correlations Between the Critical Concentration of ADP and Platelet Counts and the Maximal Release of ATP by Thrombin*

	Maximal release by thrombin	Critical concentration of ADP
Platelet counts	0.8987	0.8595
Maximal release by thrombin		0.9565

*Random sample size = 18. Error probability for all constellations < 0.001.

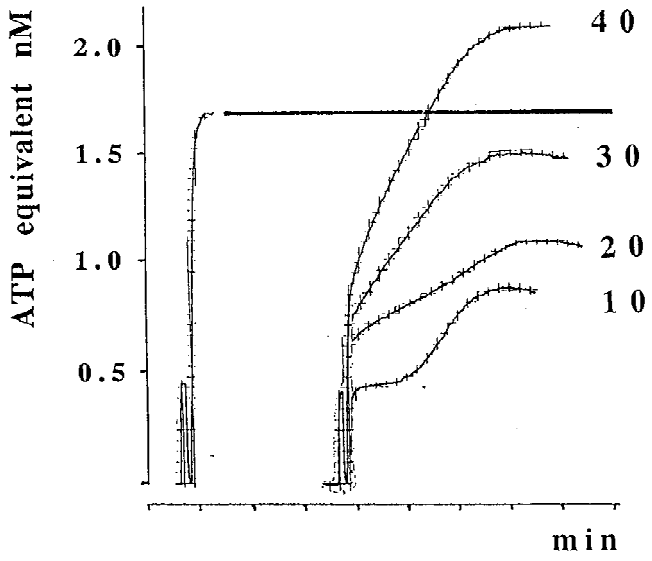

Fig. 4. A series of ATP release experiments with different concentrations of ADP (10–40 µM). The optimal ATP release by thrombin (1st curve) was taken as the standard (bold horizontal line). The release went beyond this line between ADP concentration of 30 to 40 µM. Whole blood was collected from a healthy person (platelet count 274,000/µl).

mal thrombin dose would give. Graphically this point of intersection was ascertained from curves of ATP release by different ADP concentrations. The ATP release had been calculated from the peak of the release curves and the deflection by the ATP standard. This intersection point was named the critical ADP concentration. The results of whole blood samples from 20 volunteers were subjected to correlation analysis.

The critical ADP concentration correlated only weakly with the platelet count ($r = 0.4427$, $P = 0.051$. As is demonstrated by the scatterogram (Fig. 5), the values of two patients with an extensive, postinfectious thrombocytosis (one of them suffering from a non-Hodgkin's lymphoma and the other without a well-defined basic disease) were outlyers. They were eliminated from further analysis. Without them very close correlations were found between platelet counts and critical ADP concentrations as well as between maximal ATP release by thrombin and the critical ADP concentration. The results are also given in Table I. The critical ADP concentration was determined by the equation:

$$\text{Critical ADP concentration } (\mu M) = 4.084 + 0.091 \times \text{platelet count } (\cdot 10^9/l).$$

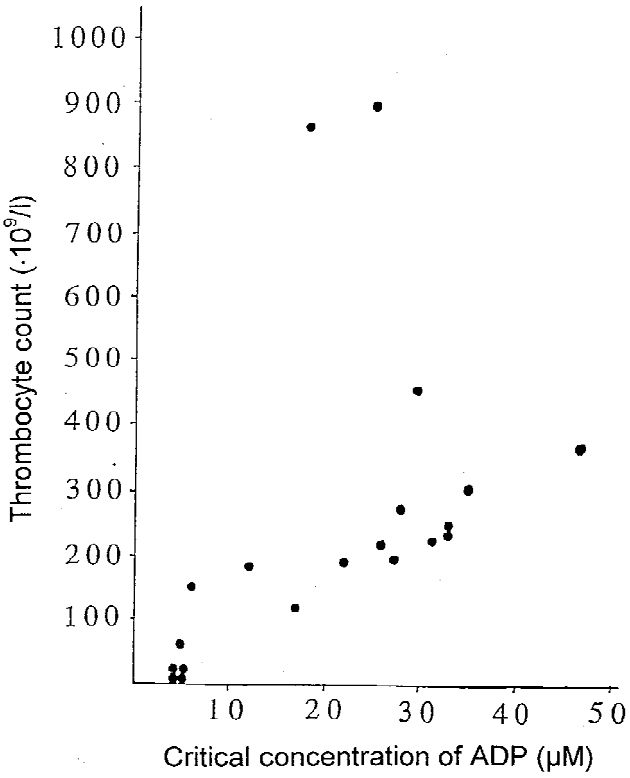

Fig. 5. Scatterogram for the relation between platelet counts and critical ADP concentrations. The values of two patients with an extreme, postinfectious thrombocytosis fell outside.

The reaction procedure without addition of ADP never was followed by release of ATP from erythrocyte suspensions. This release was not induced by arachidonic acid nor was this release induced by collagen. As was shown by the luminescence experiments in saline, the ADP preparation contained only trace amounts of ATP. The relation between ADP and ATP in the reagent was $1{:}2.2 \times 10^{-6}$. Hemoglobin was not detected in the supernatant of whole blood samples after treatment with ADP in different amounts and after different reaction time intervals, even not after stirring in the apparatus and addition of the luciferin-luciferase reagent. The lactate dehydrogenase concentration didn't increase in the supernatants of whole blood samples and of erythrocyte suspensions by the same treatment.

DISCUSSION

The release of increasing amounts of ATP by increasing concentrations of ADP in whole blood systems is described. The amounts of ATP substantially exceeded the releasable content from platelets and seemed to be very large. The release of ATP from platelets by thrombin must be considered as maximal and with ADP a weaker release should be expected. The intersection point, where the maximal release by thrombin crosses the release induced by ADP, was termed the critical ADP concentration. It depended on the platelet content of the sample, as can be demonstrated by its close correlation to the platelet count and to the maximal release by thrombin. The phenomenon could not be due to the ATP contamination of the ADP reagent used. It was only minor. According to our knowledge, this phenomenon of ATP release in whole blood by ADP, by far exceeding the releasable ATP from thrombocytes, has never been described before.

In the course of our investigation, it became clear that erythrocytes were the main source of the exceedingly high ATP release. The phenomenon was observed in thrombocytopenic samples and could be triggered by low ADP concentrations. It could be produced from suspensions of washed erythrocytes. It was found in suspensions of low temperature preserved erythrocytes and with whole blood samples from extremely leukocytopenic patients. Thus, the leukocytes could not be the main source for the high amounts of ATP.

In our system, the release of ATP from erythrocytes by ADP was actually measured. Without addition of ADP there was no release. The stirring and the luciferin-luciferase reagent cannot cause this phenomenon. It was not induced by arachidonic acid and collagen.

Joseph et al. [18] were unable to provoke an ATP release by ADP in erythrocyte suspensions. They had used another washing solution that contained phosphate and the ATP release was triggered by platelet-activating factor. Valles et al. [19] reported an increased ATP release by collagen in platelet-erythrocyte suspensions in comparison to platelet suspensions alone. The literature contains information about increasing as well as inhibitory influences of erythrocytes on collagen-induced platelet aggregation [20,21]

From the shape of the release curves it may be concluded that the release of ATP from platelets and the release from erythrocytes occur successively, but not in a strictly separate sequence. It is our impression that the first phase of the curve is caused by the release from platelets because it was observed only in samples with normal or even high thrombocyte content. Furthermore, it can be induced also by low ADP concentrations. The third phase may be the result of the release from erythrocytes and the second phase may be a summation of the declining first process and the increasing release by erythrocytes. Platelets possess receptors for ADP [22–24]. The binding sites for ADP on platelets are saturable but perhaps with different affinity [25–27]. However, according to our results, the binding sites on the platelet surface must have a higher affinity than the mechanisms on the erythrocyte surface, which are triggered mainly after their saturation.

The ATP release mechanism from erythrocytes is not

clear. Valles et al. [19] claimed that platelet nucleotides released by collagen may induce the nucleotide release from erythrocytes. Because ADP is released in a higher proportion than ATP, a passive leakage was excluded by these authors. Erythrocytes contain large amounts of ATP [28]; the content of ADP is much lower. In each volume of normal blood, the ATP content in the erythrocytes exceeded the releasable platelet ATP by about 50-fold. So the outflow of ATP by hemolysis must also be ruled out in our experiments. It could be caused by the reagents or stirring or another mechanism related to the treatment of the specimens. But evidence for it is missing. Hemoglobin was not detectable in the supernatants of the specimens treated in different manner. The detection method is very sensitive. Values in the nM range are recorded reliably. In the erythrocytes, the relation of ATP amounts to 4.05 μmol/g hemoglobin [29]. In our experiments, ATP was released in the order of 2–4 μM. So a release of 300 to 600 μM hemoglobin must be expected at the same time in the case of hemolysis, which can be ruled out by our detection system. The release of ATP may be an active process above all. But no information has existed about this release mechanism until now. Receptors for ADP on the erythrocyte membrane should be detected by binding studies.

Joseph et al. [18] observed during the ATP release by platelet-activating factor a shape change of the erythrocytes from a disc form to spherocytes. This could confirm a genuine release process. On the other hand, erythrocytes possess an adenylate kinase, which catalyzes the transformation of ADP to ATP and AMP. An open question remains, whether such large amounts of ATP can be generated in this way.

In the vicinity of thrombi, relatively high concentrations of ADP were detected [7]. Therefore, our model may truly reflect the situation in vivo. The phenomenon of the release of ATP by ADP from erythrocytes was also detected in whole blood specimens and could be reproduced with erythrocytes from all donors. Only a few objections may remain. The most important one concerns the citrate content in our model, because some nucleotidases are stimulated by divalent cations. Therefore, the phenomenon described here should be tested with different anticoagulants and other preparations of erythrocytes. Thrombocytes and leukocytes can be removed simultaneously by careful filtering.

CONCLUSION

The release of ATP from erythrocytes by ADP was detected as a constant and undeniable phenomenon. The amounts of ATP generated in our models were substantial. Therefore, the importance of this phenomenon must be discussed for the regulation of thrombosis and hemostasis as well as for some other processes. It is speculated that the effects of extracellular nucleotides, or so-called signal nucleotides, can be regulated by ATP released from erythrocyte surfaces.

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